

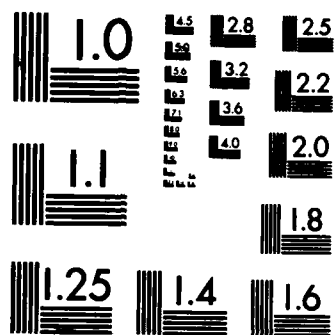
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ANNUAL REPORT

Immune Function and Psychological Stress
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Introduction

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Although the evidence for a psychosocial stress - illness relationship has been steadily growing, there has been relatively little research exploring theorized pathways for the mediation of the process. One of the more exciting prospects hypothesized as a major mechanism for translating psychological stress into subsequent illness is the immune system. The immune system is responsible for the prevention of the entry into the body of foreign bacteria, viruses, and other infectious agents, for the surveillance of, and protection from, malignancies if infectious agents gain entry, and for ridding the body of them. The immune system is comprised of a complicated protective system which produces specific antibody, cytotoxic cells, and mediators which cooperate to either prevent entry, destroy by phagocytosis, directly neutralize, lyse, or cause excretion of foreign invaders or tumors.

is focused upon

In our research we have focused on an important aspect of the immune system that plays a key role in the body's resistance by foreign agents, the secretory immune system (SIS). The SIS bathes the body's mucous membranes with secretions containing immunoglobulins. The predominant immunoglobulin of the SIS is IgA, although smaller amounts of IgG, IgM, IgD, and IgE are also present. IgA's major mechanism of action in secretory fluids seems to be through its ability to bind to foreign bacteria and viruses, preventing their adherence to the mucosal tissue of the host (Williams & Gibbons, 1972) or by direct viral neutralization (Waldman & Ganguly, 1974). Functionally, the SIS, and IgA in particular, is believed to act as the body's first line of defense against infections. IgA antibodies to a wide variety of antigens have been described in secretions (Frazekas de StGroth et al., 1950; Tomasi et al., 1965; Adinolfi et al., 1966; Tourville et al., 1968). Secretory IgA (sIgA) antibody has been shown to possess antiviral activity (Dowdle et al., 1971) and can act to prevent invasion by polio, measles, and rubella viruses (Ganguly et al., 1973; Ogra et al., 1971). Additionally, secretory IgA antibody has been able to prevent bacterial infections (Heddle & Rowley, 1975; Corbeil et al., 1974) and has been shown to be capable of bacterial toxin neutralization (Waldman & Ganguly, 1974).

continued

Although serum immunity is thought to play a role in resistance to respiratory infection (Small et al., 1971), the overriding protective mechanism in the prevention of respiratory infections seems to involve an effective secretory immunity at the oral, nasal, and respiratory mucosal surfaces (Tomasi, 1976; Shore et al., 1972; Prevost et al., 1973; Vasiljev & Alexandrova, 1972). Many studies have demonstrated little or no association between serum antibody titers and respiratory illness (Henle et al., 1946; Morris et al., 1966; Knight et al., 1965). IgA antibody levels in secretions seem to bear little relationship to serum antibody levels and, due to their continuing secretion as they are synthesized, are more susceptible to short-term alterations in level. In a recent study, the effect of academic stress on immune function as measured by the rate of secretion of salivary secretory IgA was studied prospectively (Jemmott et al., 1983). They found that the IgA protein secretion rate was significantly lower in high-stress than low-stress periods. Although these results are inter-

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esting, there were several major deficiencies in their study; first, only total immunoglobulin of the IgA isotype was measured. IgA antibody to a particular antigen is only a small fraction of total IgA and may vary quite independently of total IgA. Therefore, measurement of total IgA may not be a good indicator of an individual's current state of protection to a particular invading bacteria or virus. Secondly, the study examined IgA immunoglobulin in saliva as a function of volume of saliva (mg IgA/min). However, saliva flow is controlled by the nervous system; the simple sight of food causes increased flow (Lashley, 1916) and parasympathetic stimulation during stress causes reduced flow (Bürgen & Emmelin, 1961). It is, therefore, important to determine IgA antibody secreted not only per unit time and volume but, also relative to total IgA immunoglobulin being excreted. These parameters of IgA secretion to a particular antigen have been assessed in our research.

McClelland and his associates have conducted a series of studies examining secretory IgA in students just after they had completed an important midterm examination. Students were also seen several days later to provide estimates of various measures when they were more relaxed. The theory underlying the work is based on McClelland's formulation of personality, specifically the concepts of n Power and n Affiliation. In a representative study, two groups of students who were relatively high in n Power and n Affiliation, respectively, were exposed to a stressful task. Secretory IgA levels as measured by whole saliva collections and assayed with radial immunodiffusion methods (RID) show that sIgA was suppressed in the high n Power group two hours after the exam relative to measurements taken right after the exam (McClelland, Ross, & Patel, submitted). In a sense, this is very odd since the anticipation of the exam and the actual taking of the exam might have been expected to produce the most stress and differences between the groups would have been expected to emerge at the first measurement point. Why was suppression observed two hours later? Furthermore, these results are different than those reported by Jemmott et al. (1984) who also studied examination stress. They found that all subjects had lower IgA scores around the exam period contrasted to relatively high scores both prior to and after the examination. One of the aims of the proposed work is to replicate these results using a more controlled experimental task (the cold pressor) with RID techniques and to extend the results with ELISA assays of IgA antibody to a novel protein antigen. One of the potential problems with the RID assay is that saliva flow levels may confound sIgA levels: The ELISA technique will yield antibody levels uncontaminated by flow rate. Additionally, the levels of IgA antibody will not be affected by other possible biases such as subjects contact with individuals with respiratory illnesses.

Progress Report

A major aim of ~~our~~ ^{this} reserach during the past year was to attempt to replicate McClelland's work concerning the relationship between stress and total IgA and to compare these results with those produced by the effects of stress on IgA antibody levels in response to a specific antigen. Before undertaking this study, however, we sought to improve several aspects of our experimental procedure.

Improving the Assay We have devoted much effort to developing assays capable of detecting an antibody response to a novel antigen administered in

relatively low doses to research subjects. This has involved testing different antigens and using various methods to increase the sensitivity of the immunologic assays. In one set of preliminary studies, we orally immunized experimental subjects with a purified preparation of rabbit serum albumin at a dose of 100 mg/day/subject during a period of 15 days. Using an indirect enzyme-linked immunosorbent assay (ELISA), we assayed IgA antibody to rabbit albumin in the subjects' saliva. However, the rabbit albumin at this low dose induced IgA levels in saliva that were very low and difficult to assay reliably.

In order to solve this problem we attempted to use another, potentially more immunogenic, antigen. The antigen which we decided to examine was sperm whale myoglobin. This molecule is both from a species which is not closely related (evolutionarily) to either us or other species from which we ingest albumin and is not as evolutionarily conserved as is albumin (Atassi & Kazim, 1978) making it potentially more immunogenic. Additionally, many studies have examined antibody responses to sperm whale myoglobin (Atassi & Kazim, 1978) and human administration should be very safe since people in parts of the world (Japan, Iceland, etc.) routinely ingest it as part of their diet. We obtained recrystallized sperm whale myoglobin (Sigma Chemical Co., St. Louis) and prepared capsules containing 100mg of sperm whale myoglobin + 900 mg of NaHCO_3 to help neutralize stomach acidity and prevent premature degradation of the antigen (Goldblum et al., 1975). Four of the people who work in the laboratory volunteered to take the capsules for a period of 4 weeks. We developed an ELISA assay for the anti-sperm whale myoglobin IgA antibody in the saliva which we collected from ourselves initially using a rat serum model. The preparation of rabbit anti-rat IgG rabbit serum and goat anti-rabbit alkaline phosphatase conjugate was previously described (Cox & Muench, 1984). Activity of the antigen was confirmed by gel-diffusion analysis. Although we were able to easily determine antibody in rat serum to the sperm whale myoglobin following systemic immunization of rats (in the development of the assay), upon changing to human reagents with known activity, we could not detect any antibody activity in the human saliva of the volunteers. It may be that although sperm whale myoglobin is a relatively "good" systemic antigen, it may be a poor oral antigen, possibly because it is degraded more readily than albumin before it is absorbed across the gut wall.

In working on the sperm whale myoglobin assay we did, however, discover several procedures to improve the sensitivity of the original ELISA assay for our original antigen, rabbit albumin. The ELISA assay which we originally developed for analysis of the human IgA anti-rabbit albumin was modified in the following ways: 1) The original solid phase for the assay was vinyl which we found produced a high "background" attributable to non-specific binding of human non-antibody IgA to the plate surface. We changed this to a "low binding" formulation of styrene (Vanguard International, Neptune, N.J.) thereby allowing us to increase the concentration of rabbit albumin on the plate surface 10 fold. 2) We changed the first antibody layer (rabbit anti-human IgA) to affinity purified rabbit anti-human IgA (Jackson Laboratories, Avondale, PA). 3) We changed the secondary reagent from alkaline phosphatase goat anti-rabbit gamma globulin to affinity purified, Fc specific goat anti-rabbit IgG F(ab')_2 alkaline phosphatase conjugate (Sigma Chemical Co., St. Louis, MO) which greatly reduced the background binding. These three major changes in the ELISA assay allowed measurements of sIgA antibody to rabbit albumin to be made which are at levels which are approximately 12X less than could be detected with the previous ELISA

assay.

Recently, we have even further improved the sensitivity of our ELISA assay by utilizing the extremely high affinity binding of biotin to avidin (10^{15} 1/M). In this assay we layer rabbit albumin on a styrene plate surface as previously done. The saliva samples are applied to the plate followed by F_c specific rabbit anti-human IgA (Jackson Laboratories, Avondale, PA). Biotinylated goat anti-rabbit IgG followed by a mixture of biotinylated alkaline phosphatase (Vecto Labs, Burlingame, CA) and avidin is then added to the plate. The avidin conjugates the biotinylated anti-rabbit IgG already bound to the plate to the biotinylated enzyme. The affinity of the reaction, being much higher than that of antibody-antigen reactions, has drastically reduced non-specific binding to the plate surface. This has allowed us to measure anti-rabbit albumin IgA in samples with greatly improved sensitivity over even our modified traditional ELISA. We are now successfully using rabbit albumin as the antigen for our studies and this improved avidin-biotin ELISA in its analysis.

Mood and SIgA Associations in a Naturalistic Study Our earlier research has examined the relationship between daily mood and immunity. The design of our mood--immune study was to follow a group of dental students for approximately eight weeks by having them complete questionnaires and by collecting saliva samples three times a week. These collections began 2 weeks after the start of oral immunization with purified rabbit albumin and ingestion of albumin continued throughout the entire study. (The albumin was used to stimulate secretory IgA antibody and our assays tracked the salivary IgA response to the albumin, a process analogous to the immune response to encountering a virus, for example.) Among other psychological concepts, the questionnaires assessed daily mood using a mood adjective checklist and yielded two mood scales: positive mood and negative mood (Stone, 1981). From the saliva collections, we assayed sIgA antibody produced in response to the albumin. The analyses showed that on days with more negative mood there was less antibody compared with days with less negative mood. Days classified as high in positive mood showed higher levels of antibody compared with days classified as having less negative mood. The correlation between the positive and negative scales was only -.21, suggesting an independent contribution of each scale. Because daily stress affects daily mood (Rehm, 1978; Lewinsohn, 1974; Stone, 1981; Stone & Neale, 1984), these results demonstrate the potential for daily stress to influence sIgA antibody synthesis as well as susceptibility to respiratory illness.

Laboratory Stress and Secretory Immunity Twenty-one male undergraduate participated in this study. They came to the lab 5 times during a 21 day period. On the first visit a personal medical history was taken, heart rate and blood pressure were measured, and saliva was collected. At this time, subjects were also given a supply of capsules containing 50 mg of rabbit albumin and 450 mg of sodium bicarbonate (to neutralize stomach acidity and prevent premature degradation of the albumin). Two capsules were ingested daily, 30 minutes before breakfast, for the remainder of the study. Four to five days after the first visit or during the second visit, saliva was again collected. At the third visit, 11 or 12 days after the initial visit, the Thematic Apperception Test (TAT) was administered. The fourth visit took place 14 days after the initial visit. During this visit heart rate and blood pressure readings were taken and a stressful task, the Stroop or cold pressor, was administered. During the cold pressor task subjects were asked every 20 sec. to estimate on a scale from

1 to 100 how stressed they felt. Before the Stroop task was administered, subjects were informed that their responses would be recorded in order to detect how fast and accurately they were performing. Saliva samples were collected just before, immediately after, and three hours after either the Stroop or the cold pressor task. During the fifth visit, or the last day of the experiment, the protocol of the fourth visit was repeated with the exception that subjects who had been exposed to the cold pressor during their fourth visit, now were administered the Stroop task and vice versa.

Both unstimulated whole saliva and stimulated parotid saliva were collected. Unstimulated whole saliva was collected by having subjects spit into a cup for 3 minutes. Stimulated parotid saliva was collected by having subjects suck on a vitamin C tablet for one minute, at which time subjects swallowed the saliva in their mouth and a Lasley cup was placed over their right Steinson's duct and parotid saliva was collected for two minutes.

Two assays were used to determine secretory IgA antibody activity: radial immunodiffusion (RID) and enzyme-linked immunosorbent assay (ELISA). Neither Stroop nor the cold pressor task affected antibody level or total sIgA in parotid saliva. With regard to whole saliva, a significant decrease was observed in total sIgA right after both cold pressor and Stroop as compared with the prestress level. Antibody level in whole saliva was not affected by the Stroop task but a significant decrease was observed both right after and 3 hours after the cold pressor task.

As flow rate can affect total sIgA, total sIgA values were divided by flow rate. When this was done, the effects of the stressor on total sIgA in whole saliva was no longer evident, nor were the effects of cold pressor on antibody level in whole saliva.

It is surprising that total sIgA decreased in whole saliva but not in parotid saliva. One possible explanation is that the whole unstimulated saliva collected right after the stressors was affected by the vitamin C given right before the stressors when the stimulated parotid saliva was collected. As the stressors took only a few minutes, the vitamin C may have been still stimulating the saliva, resulting in increased flow rate which would decrease the total sIgA. Furthermore, when total sIgA was divided by flow rate, the effects of the stressors were no longer apparent. Consequently, the decrease in total sIgA appears to be due to changes in flow rate rather than immunosuppression. In fact, flow rate was higher right after the stressor than at any other point or 1.73 ml., 1.92 ml. and 1.42 ml. right before, right after, and 3 hours after the Stroop. For the cold pressor task the flow rate was 1.73 ml., 2.10 ml. and 1.80 ml., respectively.

Why antibody levels change in whole saliva after the Stroop test but not in parotid saliva is more difficult to explain. As previously mentioned, antibody levels should not be affected by flow rate. One possibility is that the small antibody response to the immunization procedure observed in parotid saliva, could have yielded a floor effect; the effects of the stressors could not be detected as there was no "room" to decrease. On the other hand, the antibody level was higher in whole saliva, which could be due to the fact that whole saliva consists of saliva from several glands rather than only one as parotid saliva does. Consequently, the effects of the stressors could be detected, as

the level of antibody could decrease. Another possibility is that the subjects did not take the albumin as instructed.

With these equivocal results, we decided against embarking on the intervention study we had described in our original proposal. Instead, we conducted another study to see if we could increase antibody response by increasing the amount of antigen ingested and also refine our stress task administration procedures. Subjects were also required to take the antigen in the presence of one of our staff so we could be sure that it was indeed being taken.

An Attempt to Increase Antibody Response Subjects were 7 male students contracted by advertisement. Rabbit albumin was administered in 100 ml of chocolate milk containing either 700 mg. or 1400 mg. of rabbit albumin and 1000 mg. of sodium bicarbonate. The milk and antigen was administered 3 times a week. Thus, one group of subjects ingested 2100 mg. of the antigen per week and another group 4200 mg. Both these doses are considerably larger than those used in our prior research. The stressors used in this study were again the Stroop and the cold pressor task.

Subjects came to the lab 13 times on Mondays, Wednesdays, and Fridays over a 30 day period. During the first visit, a personal medical history was taken, heart rate and blood pressure were measured, saliva collected and the antigen administered. During the following 11 visits subjects filled out a short form of the Perceived Stress Scale and the mood questionnaire, saliva was collected and the antigen administered. During the last visit subjects filled out the complete version of the Perceived Stress Scale and the mood questionnaire and a series of stressful tasks was administered: cold pressor task, the Stroop, cold pressor task, the Stroop. Subjects had a 5 minute break between stressors during which time the investigator left the room. During the cold pressor tasks subjects were asked every 20 sec. to estimate on a scale from 1-100 how stressed they felt. To increase stress subjects were told after the first cold pressor task that the task would be repeated later during the session and that they would be asked to keep a hand in the water for 5 minutes instead of 2 minutes. During the Stroop subjects were told that their responses would be recorded in order to detect how fast and accurately they were performing.

Saliva was collected right before the first administration of the cold pressor, during the second administration of the cold pressor, at the end of the experimental session, or right after the second administration of the Stroop task. 1 1/2 hours and 3 hours after the end of the session subjects came back to the lab and their saliva was again collected.

The immunization procedure did not work. The ELISA values were extremely close to the buffer values which measure the noise in the assay. Thus, it appears that the subjects did not develop antibody to the rabbit albumin. The reason is difficult to determine. Most likely, however, the milk could have flushed the antigen through the gut before it could stimulate Peyer's patches to begin antibody production.

Publications

Stone, A.A., Cox, D.S., Valdimarsdottir, H., Jandorf, L., & Neale, J.M. Evidence that secretory IgA antibody is associated with daily mood. Journal of Personality and Social Psychology, in press.

Stone, A.A., Cox, D.S., Valdimarsdottir, H., & Neale, J.M. Secretory IgA as a measure of immunocompetence. Journal of Human Stress, in press.

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